Effect of Immobilization Method on Activity of Alpha-Amylase

A Thesis

Presented in fulfillment of the Requirements for Graduation with Distinction in the Department of Food, Agricultural, and Biological Engineering at

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1. Introduction

Starch is a large complex carbohydrate used as a way for plants to store excess glucose. As one of the earth's most abundant sources of energy, the starch industries world-wide have experienced exceptional growth in the past decades. The corn refining in the U.S. is a $7.8 billion industry annually (U.S. Department of Commerce, 2002). Enzymes such as alpha-amylase as well as glucoamylase and pullulanase play a key role in the starch liquefaction process leading to refined syrups and sweeteners widely used in the food industry (Gerhartz, 1990). The starch refining industry spends $62.2 million each year on these enzymes (Chaplin, 1990). Immobilization allows for easy separation of the enzyme from the starch hydrolysis products which can save the enzyme, labor, and overhead costs (Gerhartz, 1990). Calcium alginate beads are widely used in enzyme immobilization because the gel formation occurs at mild conditions and poses no risk of harm to humans.

Physical entrapment of alpha-amylase in calcium alginate beads has shown to be a relatively easy, rapid and safe technique (Dey et. al, 2003). Entrapment requires that the immobilized enzyme should be a large enough molecule to be kept inside the gel matrix but the substrate and product should be small enough to pass through the pores of the gel. Calcium alginate beads made with 2% (w/v) solution have an average pore diameter of 80 to 100 Å (Stewart and Swaisgood, 1993). Starch molecules are very large, often reaching a molecular weight of 80 million Daltons (Gerhartz 1990). It is expected that starch hydrolysis reaction could occur more effectively if enzyme bound to surface.

Therefore, a further study of a comparison of different methods of immobilization of α-amylase to calcium alginate beads would provide useful information on the efficiency of the hydrolysis of starch into smaller sugars.

Process yield per unit of enzyme is improved by immobilization. In this study, alpha-amylase is immobilized by surface binding or by physical entrapment using calcium alginate gel beads. The objectives are to compare the effects of immobilization method and the bead size on the activity of immobilized alpha-amylase.

For surface immobilization, calcium alginate beads are prepared by dripping a sodium alginate solution through needles of varying gauge into a calcium chloride solution. The beads are then exposed to an alpha-amylase solution. For entrapment, a sodium alginate solution containing alpha-amylase is dripped into a calcium chloride solution to produce beads containing immobilized enzyme. For both immobilization methods, three bead sizes (2.04, 2.26, and 2.74 mm diameter) are prepared. Alginate beads containing entrapped or surface adsorbed enzyme are added to a starch solution. Starch degradation is monitored as a function of time by a starch-iodine colorimetric assay.

The initial velocities using beads with entrapped enzyme are 0.8857, 0.7261, and 0.5595 mg starch degraded / (g bead * min) for small, medium, and large beads respectively. For surface bound enzyme, initial velocities are determined to be 1.7792, 1.4485 and 1.1491 mg starch degraded / (g bead * min) for small, medium, and large beads.

The surface binding method requires less enzyme than the entrapment method to achieve the same catalytic activity. This was determined through enzyme activity analysis of the spent solutions in the surface adsorption and entrapment procedures. The results reveal an increase of enzyme activity with decreasing bead diameter. Higher enzyme activity is observed with small beads indicating the larger surface/mass ratio allows a greater amount of immobilized enzyme to have access to substrate. The surface immobilized enzyme yields higher activity per gram beads than the enzyme immobilized by entrapment because enzyme entrapped within the alginate gel-matrix is less accessible to high molecular weight starch. Thus, the combination of surface immobilization and bead size optimization should offer more efficient immobilization of alpha-amylase which leads to starch more cost-effective starch liquefaction process.

2. Literature Survey

2.1. Alpha-Amylase and Starch Liquefaction

Starch is a large complex carbohydrate used as a way for plants to store excess glucose. The hydrolysis of 1-4 glycosidic bonds (Figure 1) in starch molecules leads to a conversion of starch into simple sugars (Wiseman, 1995). As one of the earth's most abundant sources of energy, the starch industries world-wide have experienced exceptional growth in the past decades. The corn refining in the U.S. is a $7.8 billion industry annually (U.S. Department of Commerce, 2002).

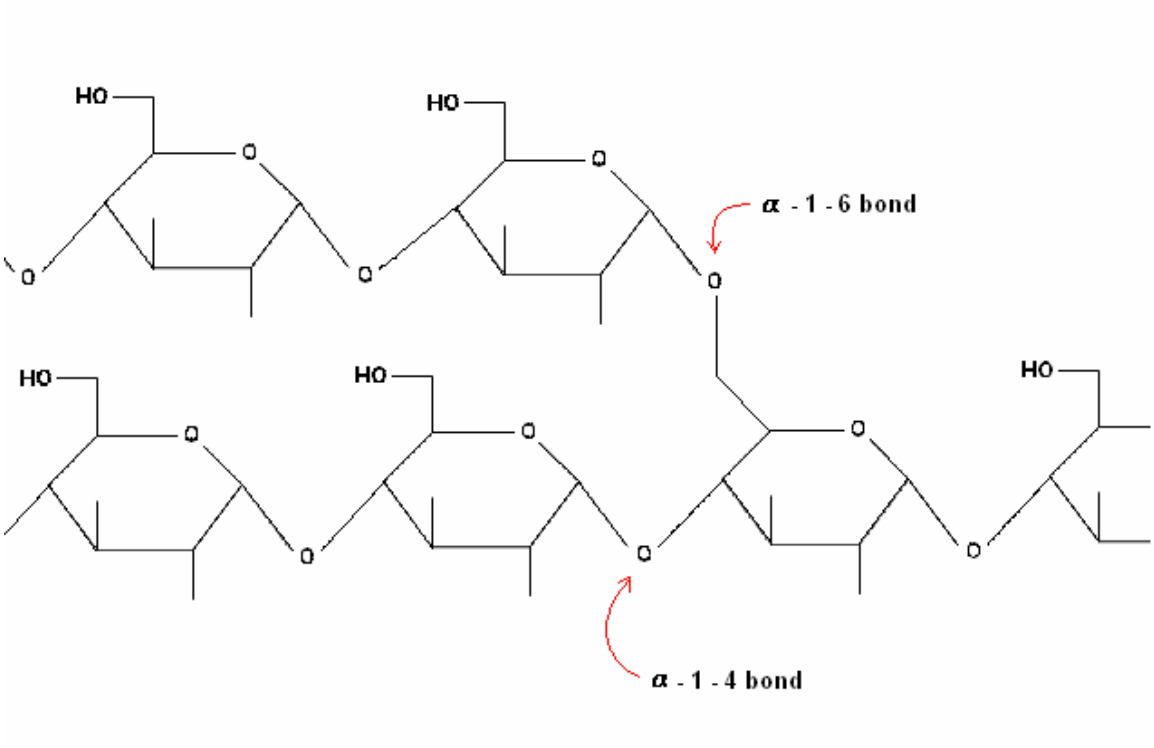

Figure 1: alpha-1-4 and alpha-1-6 glycosidic bonds in starch

Enzymes such as α-amylase play a key role in the starch liquefaction process leading to refined syrups and sweeteners widely used in the food industry. The starch refining industry spends $62.2 million each year on these enzymes (Chaplin, 1990). Immobilization allows for easy separation of the enzyme from the starch hydrolysis products which can save the enzyme, labor, and overhead costs (Gerhartz, 1990). The enzyme catalyzed degradation of starch into smaller sugars is important in the production of syrups in food industry (Figure 2). This process, which has used α-amylase as an industrial biocatalyst since the 1940's in order to thin starch to less viscous liquid and lead to syrup production, has limitations because of not being able to recover the catalyst after the process.

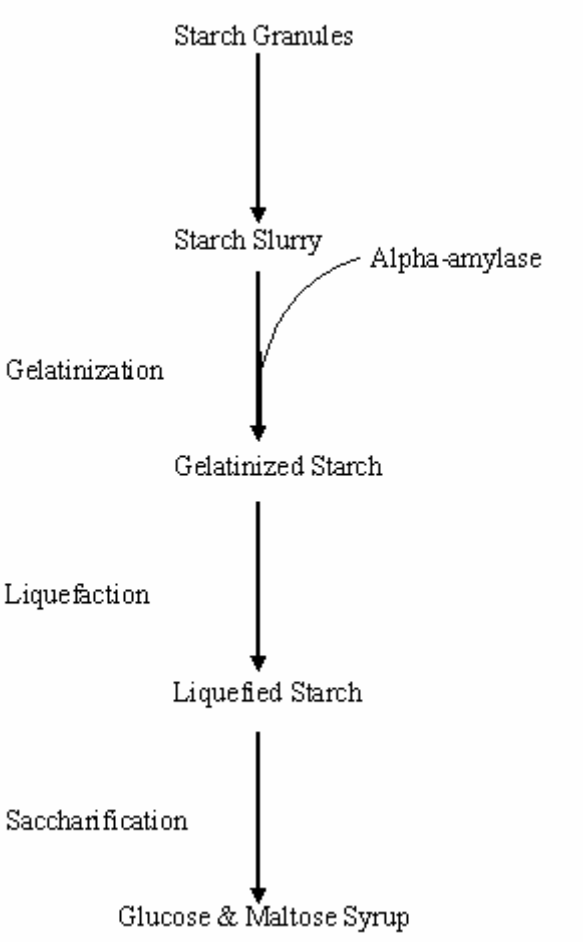

Figure 2: Flow chart of starch liquefaction process

2.2. Immobilization of Alpha-Amylase

A number of methods have been developed to immobilize α-amylase for easy recovery including entrapment within cross linked polyacrylamide gel, covalent binding to the surface of sepharose gel beads activated by cyanogen bromide (CNBr), and entrapment within calcium alginate beads (Sadhukhan et al., 1993). Among the methods, physical entrapment in calcium alginate beads has shown to be relatively easy, rapid, and safe in comparison with other immobilization methods (Bickerstaff, 1997). A comparison of

stabilization of alpha-amylase using immobilization by entrapment within polyacrylamide gel, calcium alginate beads, and covalent binding to sepharose beads demonstrated that enzyme activity of α-amylase was best maintained when the enzyme was entrapped in calcium alginate beads (Sadhukhan et al., 1993).

2.2.1. Entrapment

The preparation of alginate beads involves mixing of sodium alginate and alpha-amylase solutions in buffer, followed by dripping of the solution into calcium chloride solution to form a gel (Sadhukhan et al., 1993). Calcium alginate physical entrapment at pH 5 and at 60 °C reported to be optimum conditions for α-amylase activity immobilized by physical entrapment (Dey et al., 2003). A bead size of 2 mm produced the fastest rate of reaction when compared to calcium alginate beads of 3, 4, and 5 mm diameter (Dey et al., 2003). The physical entrapment process requires that the immobilized enzyme should be a large enough molecule to be kept inside the gel matrix but the substrate and product should be small enough to pass through the pores of the gel (Galazzo and Bailey, 1990).

2.2.2. Surface Adsorption

Alginate gel has also been used for purification of α-amylase enzyme from a mixture of proteins encountered in downstream processing (Sardar and Gupta, 1998). Their results showed that α-amylase has an affinity for alginate and selectively binds to alginate gel. This process has been used in the past for the purification of alpha-amylase, but never for immobilization for starch hydrolysis. This process is most often used with covalent cross linkage to adhere the enzyme to the calcium alginate bead surface.

2.3. Objectives

The objectives of this study are:

- To compare the immobilized enzyme activity of alpha-amylase using entrapment and the surface adsorption methods
- To determine the effect of particle size on immobilized enzyme activity.

2.4 Hypothesis

The hypothesis of the study is that the immobilized enzyme prepared by surface binding will produce higher activity yield per enzyme added than entrapment because surface immobilized enzymes will be able to access the alpha-1-4 bond sites on the starch molecules more readily than enzymes entrapped within the calcium alginate gel matrix. The smallest alginate beads for enzyme immobilization will yield the highest activity because surface area per unit mass of smaller beads will be larger than that of larger beads.

3. Materials and Methods

3.1. Materials

Starch from potato (Sigma, St. Louis, MO) was used for all starch solutions. Starch solution was prepared as 0.4% (w/v) using 50 mM acetate buffer and including 6.7 mM sodium chloride for ionic stability and 5 mM calcium chloride for alginate stability at pH 5.6. Alpha-amylase from *Bacillus licheniforms* (Sigma) was used for all enzyme tests. Sodium alginate was dissolved in acetate buffer without calcium chloride at pH 5.6 to form a 2% (w/v) solution. Hydrochloric acid (1 N) was diluted to 0.5 N using nano-purified water which was then used to stop the starch hydrolysis reaction by alpha-amylase. Iodine solution was added to the reaction medium to create a starch-iodine colloid which would be used in measuring the amount of starch present in the sample. Three gauges of hypodermic needle were used from (Fisher Scientific, PrecisionGlide Needles, Pittsburgh, Pennsylvania) gauge 26, 21, 18, with inside diameters of 0.241, 0.495, and 0.838 mm, respectively.

3.2. Free Enzyme Studies

Free enzyme studies were conducted to determine enzyme activity as a function of starch concentration. Kinetic parameters, maximum velocity (Vmax), and Michaelis-Menten constant (Km), were evaluated by fitting the collected data to Michaelis-Menten kinetics model. The kinetic constants were used to assure that the starch concentration is below the experimental Km value for the free enzyme. This would assure that tests were conducted in the first order region of kinetic activity of alpha-amylase.

3.2.1. Preparation of enzyme solution

Freeze-dried alpha-amylase (Sigma) were combined with 50 mM acetate buffer solution pH 5.6 to the desired concentration 0.3922 mg solids/mL. This enzyme stock solution was stored at 4°C to be diluted for future tests.

3.2.2. Enzyme Assay

Starch solution and buffer were added to a 15 mL beaker containing a magnetic stir bar over a stirring plate. Alpha-amylase solution was added to the reaction beaker at which time a stopwatch was started. A 200 micro liter sample was removed from the stirred reaction beaker at 10 second intervals. The 200 micro liter samples were immediately added to a 10 mL test tube containing 1 mL of 0.5N hydrochloric acid to stop the reaction. Each sample was then mixed in the test tube and a 200 micro liter sample extracted and placed into a clean 10 mL test tube. To this sample was added 5 mL iodine solution. The absorbance of the starch-iodine solution was measured at 620 nm using a spectrophotometer (Varian, Cary 5000, Walnut Creek, California) after 15 minutes. Figure 3 shows the sampling and dilutions for the enzyme assay prior to absorbance measurement.

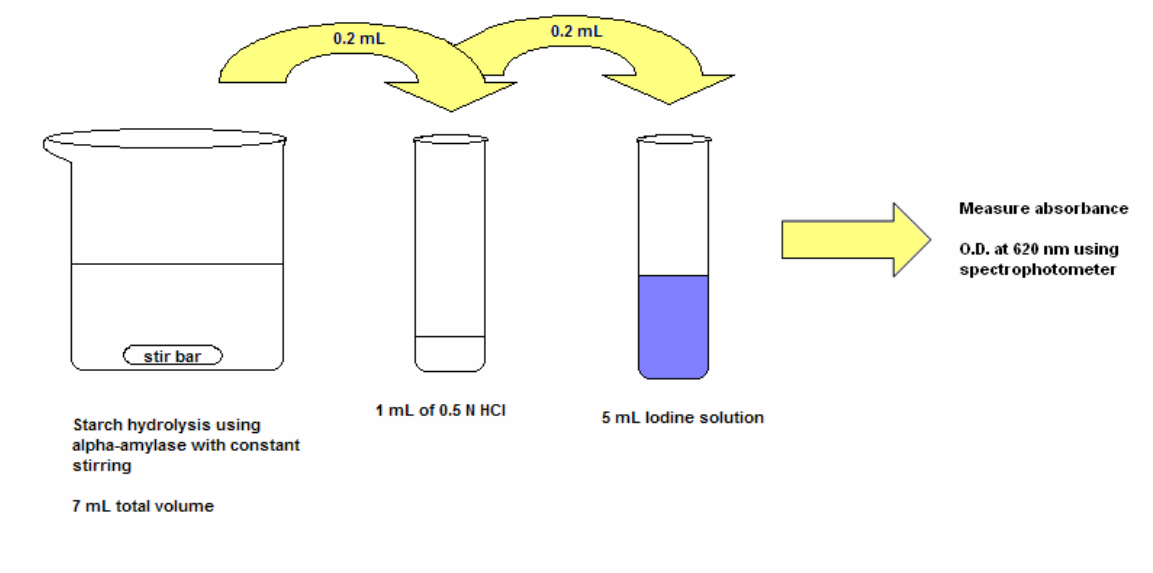

Figure 3: Enzyme assay sampling steps used for colorimetric determination of starch concentration for each sampling time interval

3.2.3.1. Starch Calibration Curve

A starch calibration curve was created to relate the absorbance reading (O.D. at 620 nm) to starch concentration (mg/mL) in the medium (Figure 1, Appendix A). Five starch concentrations (2 mL) ranging from 0 mg/mL to 0.57 mg/mL were placed in 10 mL test tubes. A 200 micro liter sample of each starch concentration was added to a 10 mL test tube containing 1 mL 0.5 N hydrochloric acid. From this solution, a 200 micro liter sample was taken and added to 5 mL of iodine solution. The absorbance of each sample was measured after 15 minutes. The absorbance (O.D. at 620 nm) is plotted as a function of starch concentration. Data are fitted to a linear equation to obtain calibration curve. This equation was used to convert absorbance readings to starch concentration for each set of relevant experiments.

3.2.3.2. Enzyme Calibration Curve

An enzyme calibration curve was created to relate initial velocity (mg/(mL*min)) to enzyme concentration (mg/mL) at a constant starch concentration (0.57 mg/mL) achieved by using 1 mL of 0.4% (w/v) starch solution in a 7 mL total reaction environment (Figure 2, Appendix A). This was used to then determine the amount of enzyme present in a given solution based on the initial velocity of that reaction with 0.57 mg/mL initial starch concentration. The enzyme concentrations were varied from 1 to 0.0392 mg solids/mL and the initial velocities measured for each. The initial velocity was plotted as a function of enzyme concentration. Data are fitted to a linear equation to use as a calibration curve.

3.3. Immobilized Enzyme studies

3.3.1. Surface Adsorption Method

3.3.1.1. Preparation of Alginate Beads

Calcium alginate beads were prepared using 2% sodium alginate solution. The alginate was dripped through a hypodermic needle using a peristaltic pump (Fisher Scientific, Variable-Flow Peristaltic Pump Low Flow, Pittsburgh, Pennsylvania) with silicon tubing, producing a constant flow rate and uniform calcium alginate bead size. The sodium alginate was dripped into a stirred beaker of 0.1 M calcium chloride solution as shown in Figure 4. The beads were incubated at room temperature for 1.5 hours in 0.1 M calcium chloride to harden. The liquid was removed using a ceramic Hirsch funnel (Fisher Scientific, 10-360-11, Pittsburgh, Pennsylvania) to separate calcium alginate beads from

solutions. After separation, the beads were equilibrated in 50 mM acetate buffer containing 6.7 mM sodium chloride and 5 mM calcium chloride at pH 5.6.

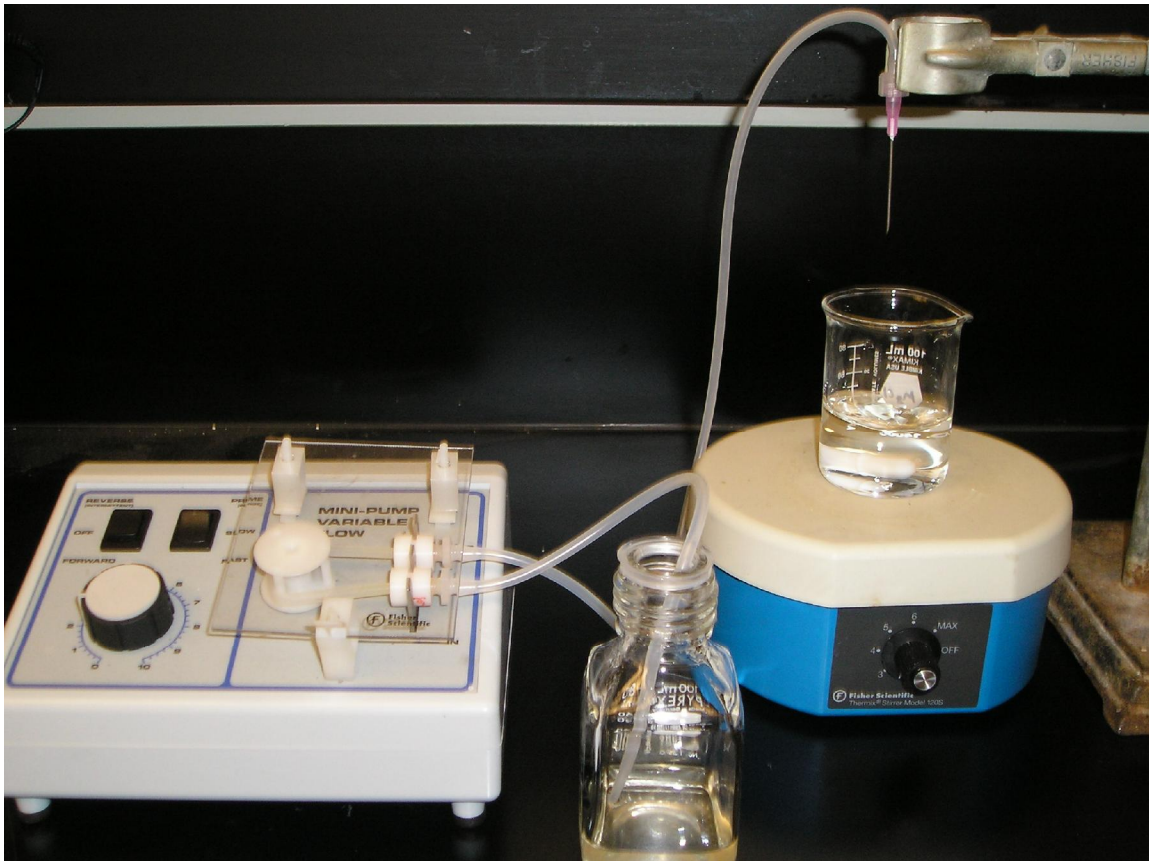

Figure 4: Pump configuration for production of calcium alginate beads

3.3.1.2. Preparation of Adsorbed enzyme

The free enzyme activity of the initial alpha-amylase solution (0.0392 mg solids/mL) was measured and checked against the enzyme calibration curve to verify the enzyme concentration. The enzyme solution (10 milliliters) was combined with 1 gram of calcium alginate beads and 5 mL of acetate buffer in a beaker with constant stirring. The enzyme and beads were incubated for 1.5 hours at 25°C. The beads were removed from the used enzyme solution using a ceramic funnel. The funnel was rinsed with 1 mL acetate buffer

to be combined with the used solution. The beads were combined with 10 mL acetate buffer under constant stirring to rinse for 15 minutes. The beads were separated using a ceramic funnel. The funnel was rinsed with 1 mL acetate buffer which was combined with the wash solution. One gram of alginate beads containing alpha-amylase were combined with 1 mL 0.4% starch (w/v) and 6 mL acetate buffer in a 15 mL beaker constantly stirred. Samples (200 micro liters) were extracted from the reaction medium at timed intervals for 10 minutes and each sample's starch concentration was measured using the enzyme assay. Samples from the used and wash solutions were measured for enzyme activity using 1 mL of 0.4% starch in a total reaction environment of 7 mL. Figure 5 (right side) shows the schematics of surface adsorbed alpha-amylase.

3.3.2. Entrapment Method

3.3.2.1. Preparation of Entrapped Enzyme

Alpha-amylase powder (0.0032 g) was combined with 10 mL 2% sodium alginate prepared with 50 mM acetate buffer pH 5.6. The enzyme concentration in the solution was 0.245 mg solids/mL alginate solution which led to enzyme concentration of 0.172 mg solids/gram beads because the gel matrix formation caused a conformational change leading to a 0.7 ratio of grams of beads produced from 1mL of sodium alginate solution. The solution was mixed well to form homogeneous solution. The solution was dripped through a hypodermic needle using a peristaltic pump into a pre-weighed beaker of 10 mL of 0.1 M calcium chloride with constant stirring. The mass of the beaker with

alginate beads was measured. The alginate beads were incubated at room temperature in 0.1 M calcium chloride for 1.5 hours to harden. The beads were separated from the 0.1 M calcium chloride solution, called used solution, using a ceramic funnel. The funnel was rinsed with 1 mL 0.1 M calcium chloride and combined with the used solution. They were then added to 10 mL of 50 mM acetate buffer with 6.7 mM sodium chloride and 5 mM calcium chloride pH 5.6 with constant stirring for 15 minutes to rinse. The beads were separated from the wash solution using a ceramic funnel. The funnel was rinsed with 1 mL acetate buffer solution and combined with remaining wash solution. The alginate beads containing alpha-amylase (1 gram) were combined with 1 mL 0.4% starch (w/v) and 6 mL acetate buffer in a 15 mL beaker constantly stirred. Samples (200 micro liters) were extracted from the reaction medium at timed intervals for 10 minutes and each sample's starch concentration was measured using the enzyme assay. Samples from the used and wash solutions were measured for enzyme activity using 1 mL of 0.4% starch in a total reaction environment of 7 mL resulting in initial starch concentration of 0.57 mg/mL. Figure 5 (left side) shows the schematic of entrapment of alpha-amylase in calcium alginate beads.

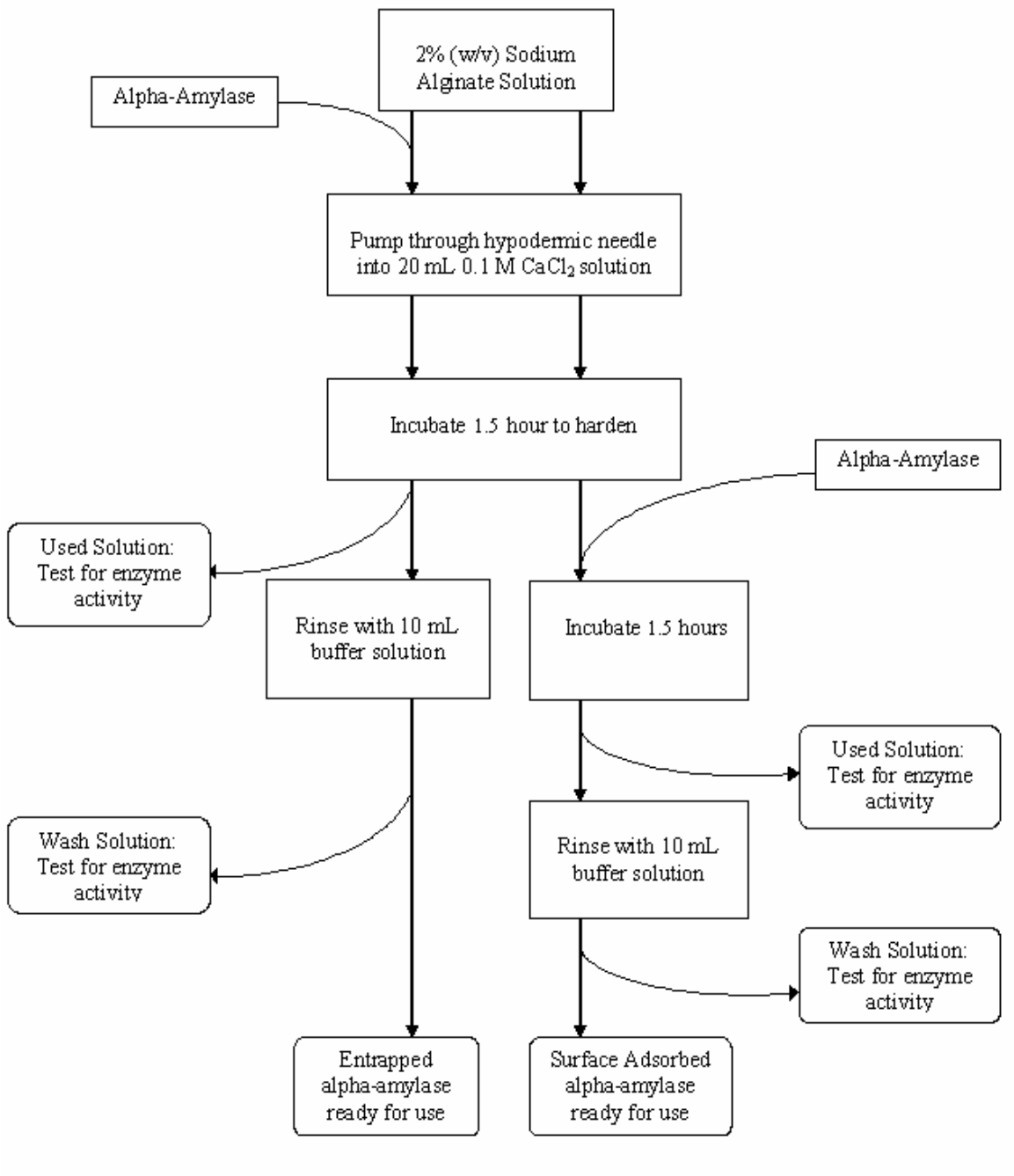

Figure 5: Flow-diagram of immobilization of alpha-amylase using calcium alginate beads by entrapment and surface adsorption

3.3.3. Effect of Bead Size

Alginate gel beads were prepared dripping sodium alginate solution with or without alpha-amylase through three gauges of hypodermic needles (18, 21, 26 gauge). The resulting beads, shown in Figure 6, are 2.74, 2.26, and 2.04 mm diameter, respectively. The percent of enzyme loaded and the percent relative activity of the beads was measured for each of the bead sizes to determine the optimum bead diameter for immobilized alpha-amylase.

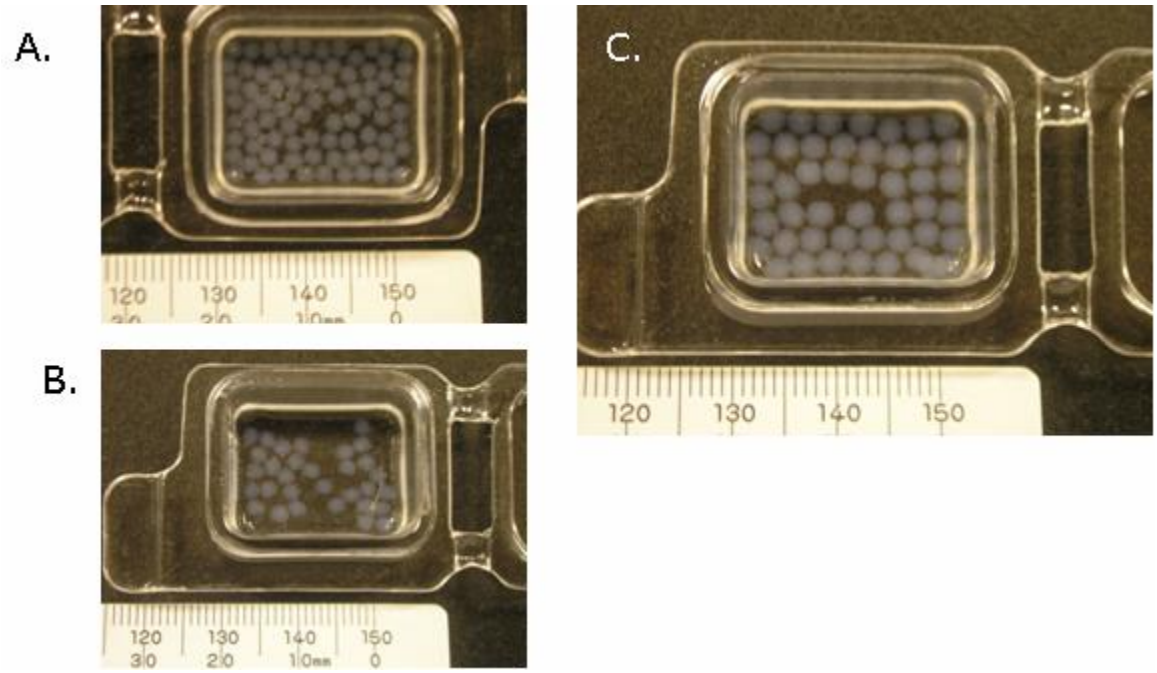

Figure 6: Calcium alginate beads prepared using three hypodermic needles, gauge 26(A), 21(B), and 18(C)

3.4. Data Analysis

3.4.1. Determination of Initial Velocities

Absorbance data collected were converted to starch concentration by using starch calibration curve. The initial velocity of the alpha-amylase degradation of starch reaction was determined from the slope of the initial linear region of the starch concentration versus time curve. As many data points as possible were used to construct the linear region such that the sum of error squared generated was a value greater than 0.98. The graph in Figure 7 shows the data points collected and those used to determine initial velocity.

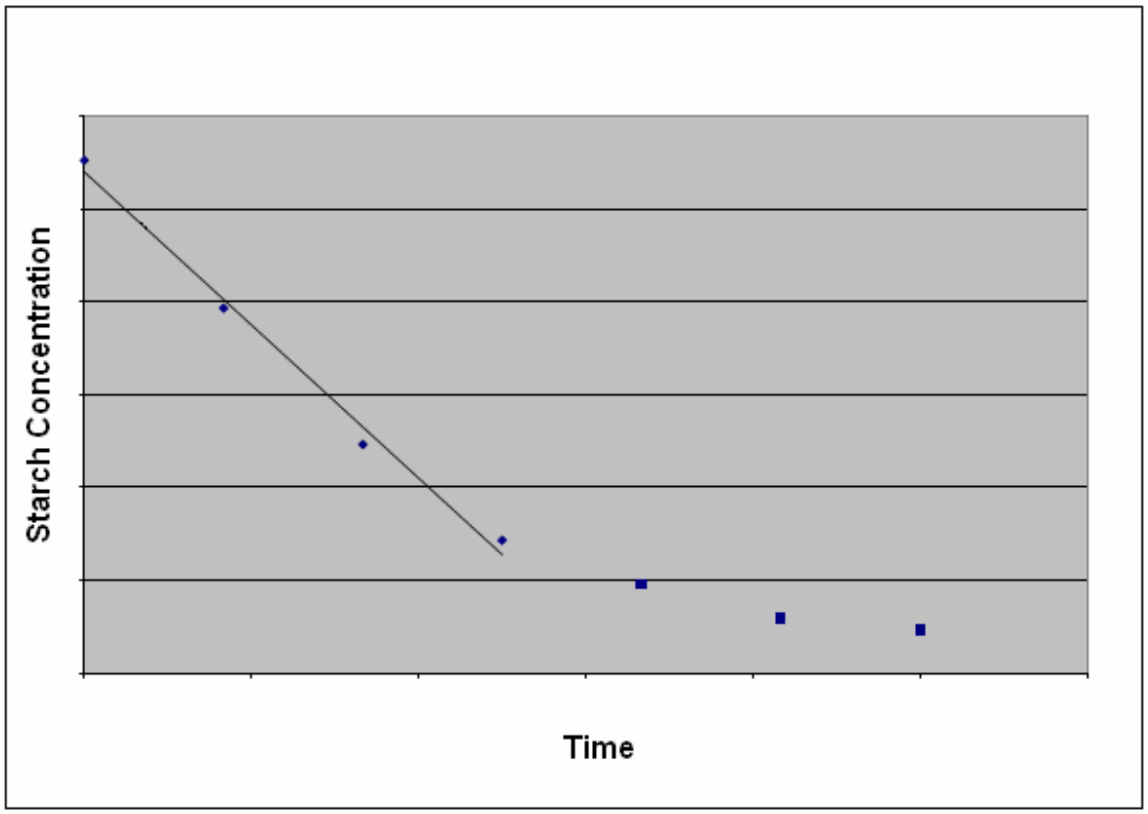

Figure 7: Graphical representation of starch concentration as a function of time with initial velocity measured

3.4.2. Kinetic Constants

The kinetic constants Km and Vmax were found experimentally by conducting a series of tests with varying initial starch concentrations from 25 mg/mL to 0.5 mg/mL. The data was fit to the Michaelis-Menten model as shown in Figure 8. The Michaelis-Menten curve models enzyme kinetics following the equation:

$$V = \frac{Vmax \cdot [S]}{Km + [S]}$$

Where V is the initial velocity (mg starch degraded/(mL*min)), Vmax is the maximum velocity achievable with the given concentration of enzyme, [S] is the initial starch concentration (mg/mL), and Km is the Michaelis constant with describes the enzyme's affinity for the substrate.

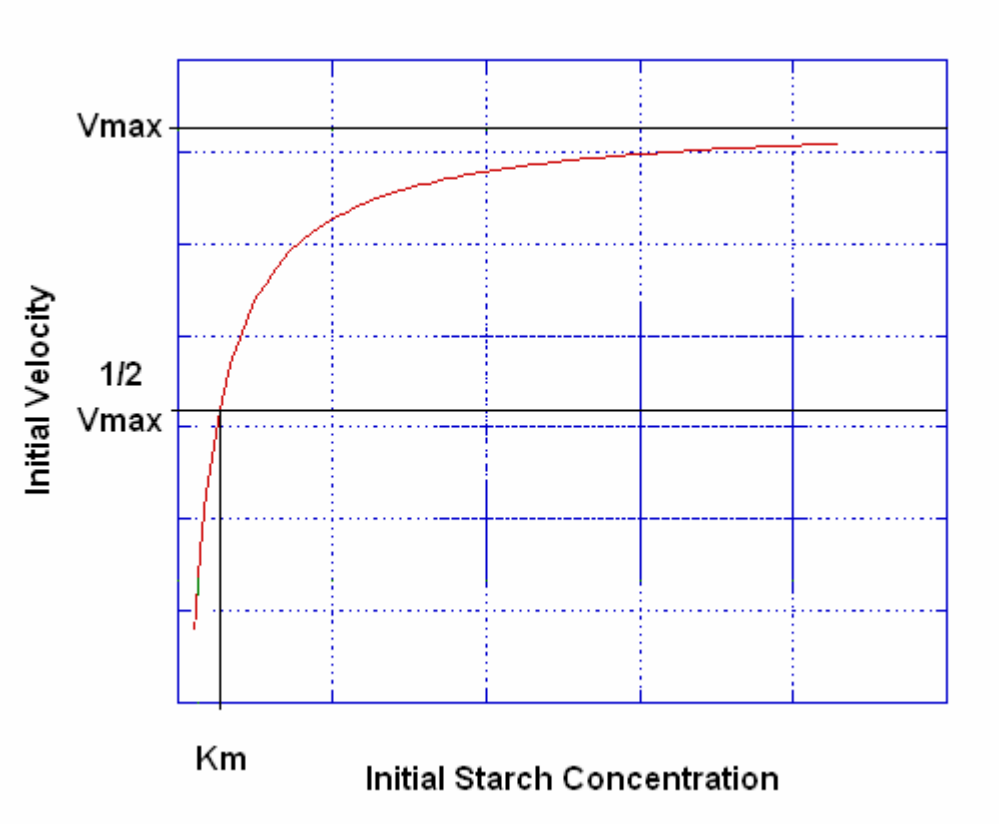

Figure 8: Michaelis-Menten enzyme kinetics model

3.4.3. Enzyme Bound to Beads

3.4.3.1. Surface Adsorption

A mass balance was applied to determine the amount of enzyme loaded on the calcium alginate bead. The initial amount of alpha-amylase introduced was determined from the activity measured from the initial enzyme solution. This is the total enzyme introduced. The enzyme activity calculated from the used and wash solutions were converted to total enzyme using the total volume of the used and wash solutions, respectively. The difference between the total enzyme introduced and the enzyme in the used and wash

solutions is the total enzyme loaded on calcium alginate beads. The initial velocity measured from the surface immobilized alpha-amylase was measured. This initial velocity value was converted to enzyme concentration. The measured amount of enzyme with respect to the calculated enzyme immobilized was called the percent relative activity.

3.4.3.2. Entrapped Enzyme

A mass balance was applied to determine the amount of enzyme entrapped within the calcium alginate gel matrix. The initial amount of alpha-amylase introduced was determined from the mass change of the beaker of 0.1 M calcium chloride. The mass of alginate was converted to volume using the density of sodium alginate 2% found to be 1.008 g/mL. The concentration of alpha-amylase in sodium alginate prepared was 0.3922 mg solids/mL. This is the total enzyme introduced. The enzyme activity calculated from the used and wash solutions were converted to total enzyme using the total volume of the used and wash solutions, respectively. The difference between the total enzyme introduced and the enzyme in the used and wash solutions is the total enzyme entrapped within the calcium alginate beads, referred to as percent enzyme loaded.

4. Results and Discussion

4.1. Free Enzyme

The data collected from free enzyme tests were fitted to the Michaelis-Menten equation to determine the Km and Vmax constants of alpha-amylase (Figure 9).

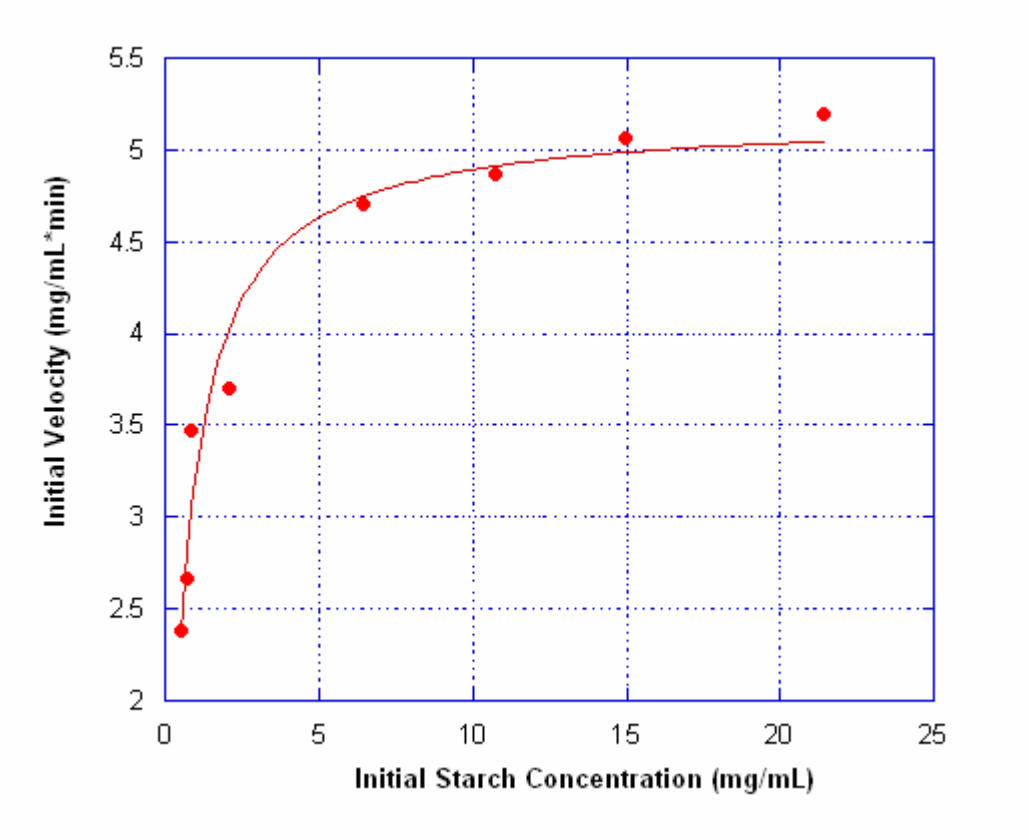

Figure 9: Michaelis-Menten direct fit

The tests yielded a Km of 0.597 mg/mL and Vmax of 5.186 mg/(mL*min). The results were used to keep the initial starch concentration below 0.597 mg/mL. A starch solution of 1mL of 0.4% (w/v) was added to 7mL total reaction medium volume to achieve a 0.57mg/mL initial starch concentration. All tests with initial starch concentrations below the Km value were conducted to assure to be in the linear first-order region of the

Michaelis-Menten kinetics model. The enzyme activity can be easily predicted with respect to temperature by testing in this region.

4.2. Immobilized Enzyme

4.2.1. Entrapped

The initial velocity (Vo_{ent}) of the starch hydrolysis reaction by immobilized alpha-amylase using entrapment method is calculated by constructing a tangent to the curve at time equal to zero (Figure 10).

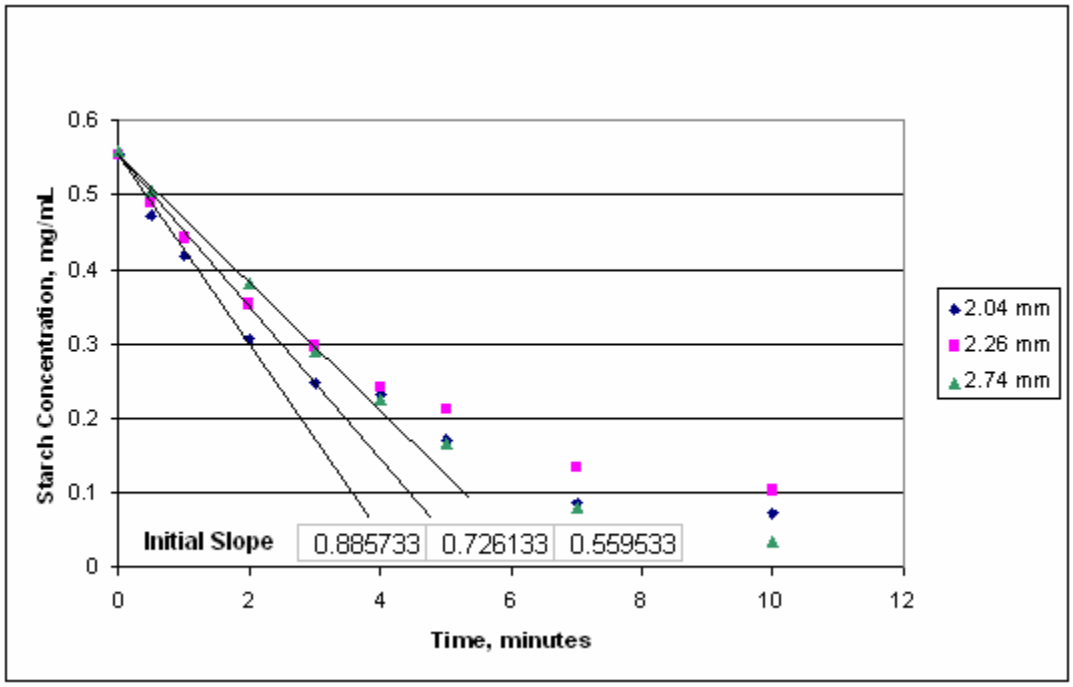

Figure 10: Starch hydrolysis using entrapped alpha-amylase in calcium alginate beads of varying diameters

Figure 10 shows the effect of bead diameter on the rate of starch hydrolysis using immobilized enzyme by entrapment. The tangent lines to the curve represent the velocity at time equal to zero. The results reveal that initial rate of the reaction decreases with increasing bead diameter.

A comparison of activity of immobilized enzyme, its relative activity to free enzyme and enzyme loaded for entrapped enzyme is given in Table 1.

Table 1: Entrapped alpha-amylase data after three trials

Bead Diameter (mm)	2.04	2.26	2.74
Vo_{ent} (mg/g bead*min)	0.8857 ± .035	0.7261 ± .037	0.5595 ± .008
Relative Activity (%)	2.089 ± .0007	1.704 ± .0008	1.349 ± .0003
Enzyme Loading (%)	46.94 ± .039	38.15 ± .11	37.97 ±.044

***** Relative Activity is Immobilized Activity/Free Enzyme Activity

The entrapped alpha-amylase shows a decrease in relative activity with increasing bead diameter. The comparison of percent relative activity of entrapped enzyme with bead size shows that while the same amount of enzyme has been added to 1 gram of beads of each diameter, the 2.04 mm beads retained about 2% of the free enzyme activity while the 2.74 mm beads retained only 1.35% of the free enzyme activity. This result could be due to decreased enzyme loading with increasing particle size. The enzyme loading reflects the amount of enzyme bound to the beads relative to the total enzyme introduced. Since

entrapment allows for binding throughout the entire calcium alginate gel matrix, enzyme loading is normalized by the bead volume for better comparison of the binding efficiency values (Figure 11).

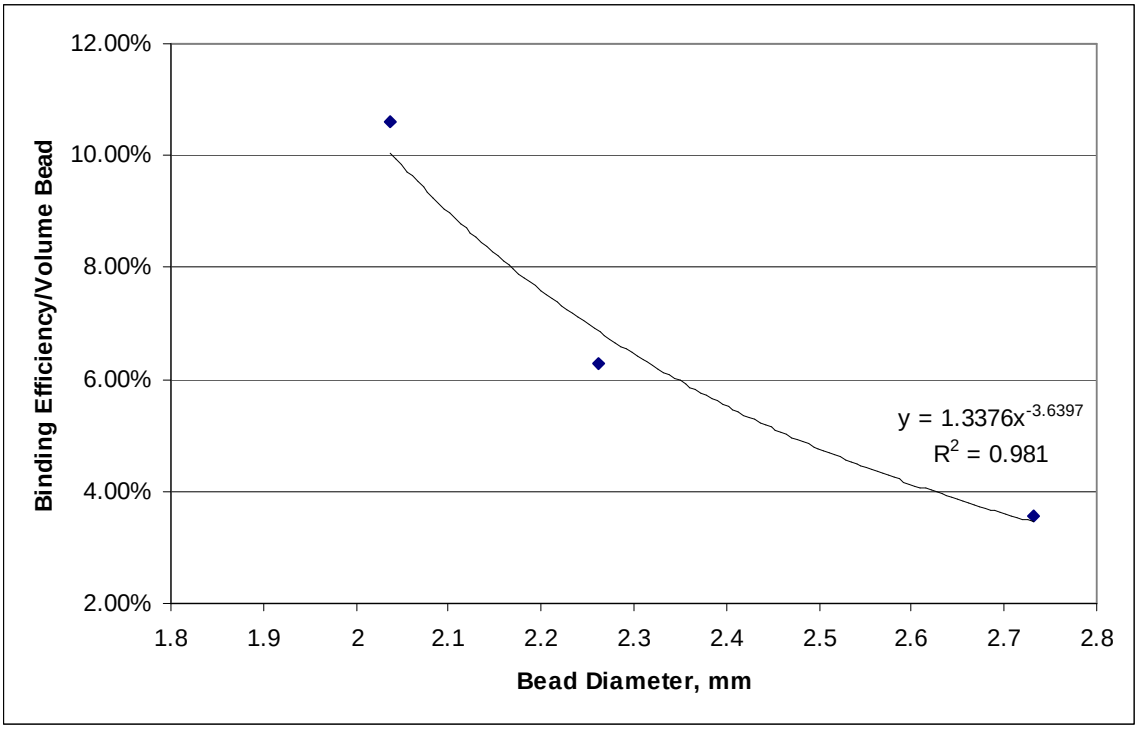

Figure 11: Enzyme loading of entrapped enzyme normalized by bead volume

Binding efficiency normalized by bead volume decreases with an increase in bead diameter (Figure 11). The relationship between binding efficiency/bead volume and bead diameter shows high dependence on particle size, even for the small range of bead diameters (2.04-2.74 mm). It also appears that binding efficiency/bead volume of entrapped enzyme levels off with increased bead size eventually. Extrapolation based on the fitted correlation suggest that binding efficiency/bead volume will reach a final average value of 1.3 percent

4.2.2. Surface Adsorption

Alpha-amylase activity analysis for the surface adsorption method of immobilization was conducted using the initial velocity which reports the amount of starch degraded (mg) per gram of beads per minute (Figure 12). Using the surface adsorption method of immobilization there is a decrease in the measured enzyme activity compared to the free enzyme. Surface adsorption resulted in higher activity than the entrapped method of immobilization.

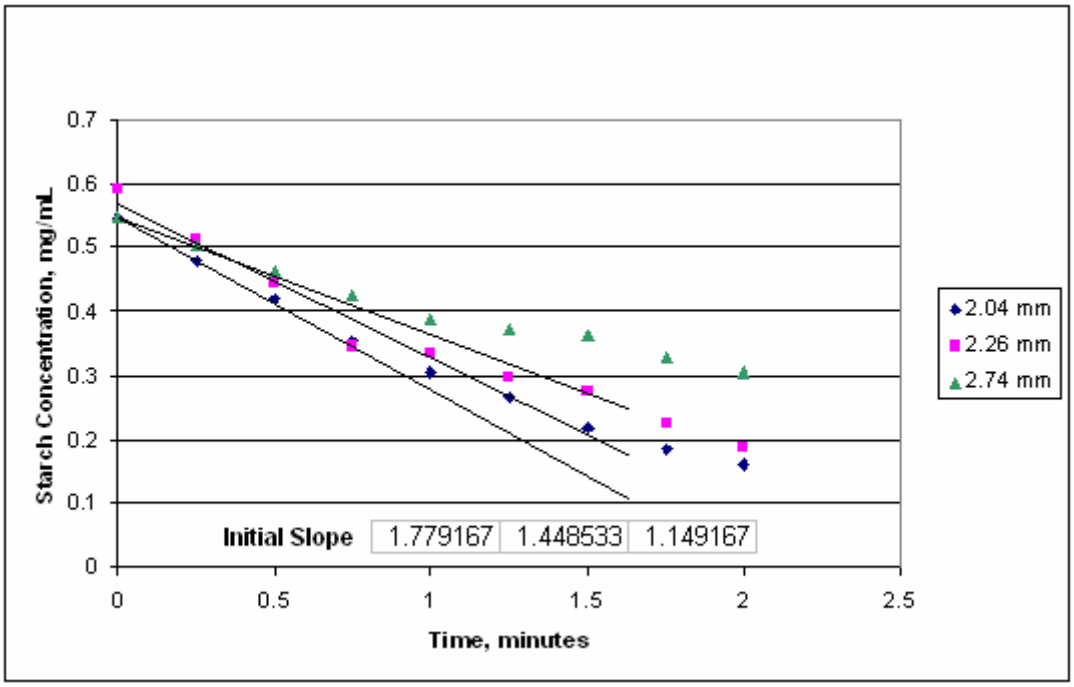

Figure 12: Starch hydrolysis using surface adsorbed alpha-amylase on alginate beads of varying diameter

Figure 12 shows the effect of bead diameter on the rate of starch hydrolysis using immobilized enzyme by adsorption. The tangent lines to the curves represent the velocity at time equal to zero (Vo_{ads}). The results reveal that initial rate decreases with increasing

bead diameter, showing the importance of exposed surface area on the starch hydrolysis reaction.

A comparison of activity of immobilized enzyme, its relative activity to free enzyme and enzyme loading for surface adsorbed enzyme is given in Table 2.

Table 2: Surface adsorbed alpha-amylase data after three trials

Bead Diameter (mm)	2.04	2.26	2.74
Vo_{ads} (mg/g bead*min)	1.7792 +/- .068	1.4485 +/- .074	1.1492 +/- .040
Relative Activity (%)	5.357 +/- .0046	4.685 +/- .0023	3.800 +/- .0018
Enzyme Loading (%)	64.46 ±.0028	60.74 ± .081	68.76 ± .023

***** Relative Activity is Immobilized Activity/Free Enzyme Activity

The adsorbed alpha-amylase shows a decrease in relative activity with increasing bead diameter. The comparison of percent relative activity of entrapped enzyme with bead sized shows that while the same amount of enzyme has been added to 1 gram of beads of each diameter, the 2.04 mm beads retained 5.3% of the free enzyme activity while the 2.74 mm beads retained only 3.8% of the free enzyme activity. The lowest relative activity from surface adsorption (2.74mm, 3.8%) is higher than the highest relative activity of entrapped enzyme (2.04mm, 2.089%). The surface adsorbed alpha-amylase show a fluctuation in binding efficiency as the particle size increases. The binding

efficiency reflects the amount of enzyme bound to the beads relative to the total enzyme introduced based on enzyme activity of spent solutions. Since surface adsorption allows for binding only on the surface of the calcium alginate beads, binding efficiency is normalized by the bead surface area for better comparison of the binding efficiency values (Figure 13).

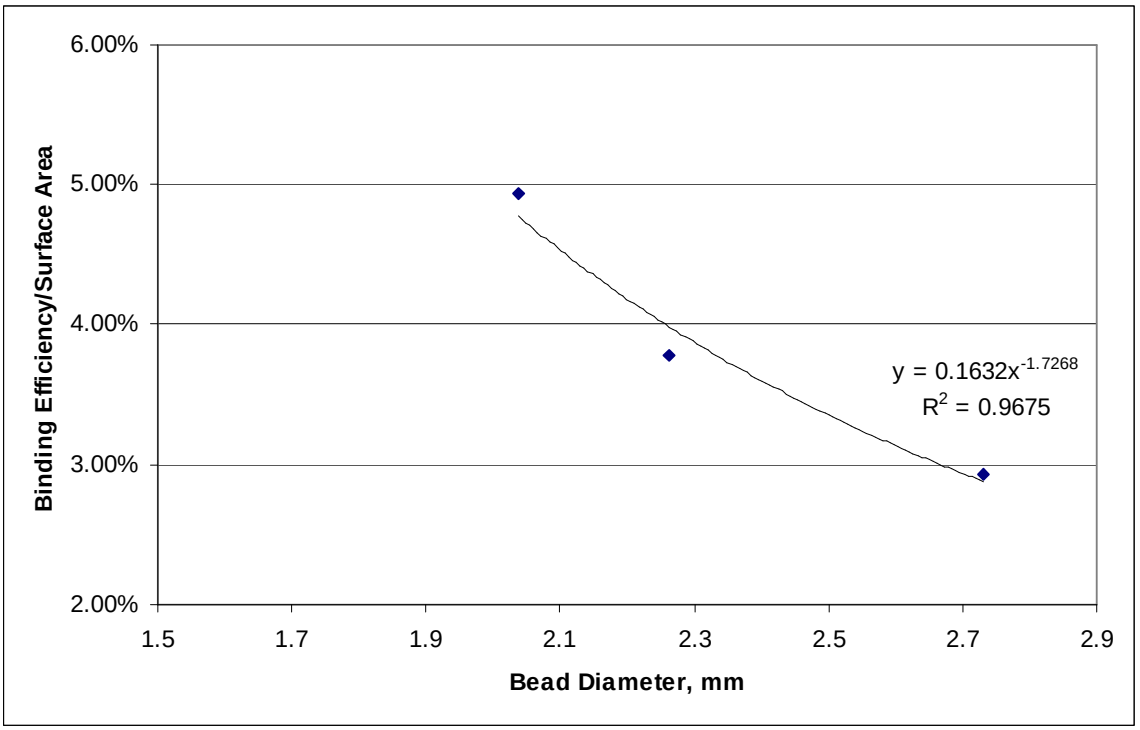

Figure 13: Binding efficiency of surface adsorbed enzyme normalized by bead surface area

The binding efficiency/bead surface area decreases as the bead diameter increases (Figure 13). The range of bead diameters tested is rather small, but the trend shows that surface adsorption binding efficiency/surface area is less dependent on bead diameter than entrapped alpha-amylase binding efficiency/volume. While the range of binding efficiency/surface area for adsorbed is about 3% for largest bead to 5% for smallest, entrapment binding efficiency/bead volume is about 4% for largest to 11% for smallest. It

is difficult to compare enzyme loading of entrapment and surface adsorption directly because the physical immobilization methods are different. Entrapped enzymes are physically bound within the calcium alginate gel matrix while surface adsorbed enzymes are attached to the bead surface through some affinity. The structural basis for alginate recognition of alpha-amylase is not clear (Sardar and Gupta, 1998). This means the potential number of binding sites differs; entrapment binding is strongly dependent on total bead volume while adsorption depends on surface binding sites, thus bead surface area. With enzyme loading normalized by bead volume for entrapment and surface area for adsorption, the data suggests that small beads with entrapped alpha-amylase load enzyme most efficiently. The largest bead with entrapped enzyme also showed slightly greater enzyme loading/volume (2.74mm, 3.96%) than the largest bead with adsorbed enzyme (2.74mm, 2.94%). Although entrapment shows greater enzyme loading than surface adsorption, the relative activity yield from the beads proves that entrapped enzymes are not participating in the starch hydrolysis reaction as readily as the adsorbed enzymes.

The relative activity of adsorbed enzyme is higher than the relative activity of entrapped enzyme which corresponds to the fact that more enzymes were immobilized by entrapment. This proves that some of the internally entrapped enzymes are not active in the starch hydrolysis reaction. More enzyme is immobilized with surface adsorption and the relative activity is higher than the relative activity measured from entrapment. The entrapment method provides more binding sites, theoretically, however.

4.2.3. Effect of Bead Size

The relative activity for immobilized enzymes produced by both methods with respect to free enzyme activity increases with an increasing surface area/volume ratio (Figure 14). Surface area/volume ratio is inversely proportional to the bead diameter. The smallest beads correspond to the highest surface area/volume value. The observed trend indicates that the surface area of the beads participates in the starch hydrolysis even if enzyme is immobilized within the alginate gel matrix. The dependence of relative activity on surface area/volume is greater for surface adsorbed enzyme than entrapped enzyme based on the slopes of the lines fitted to the data (Figure 14).

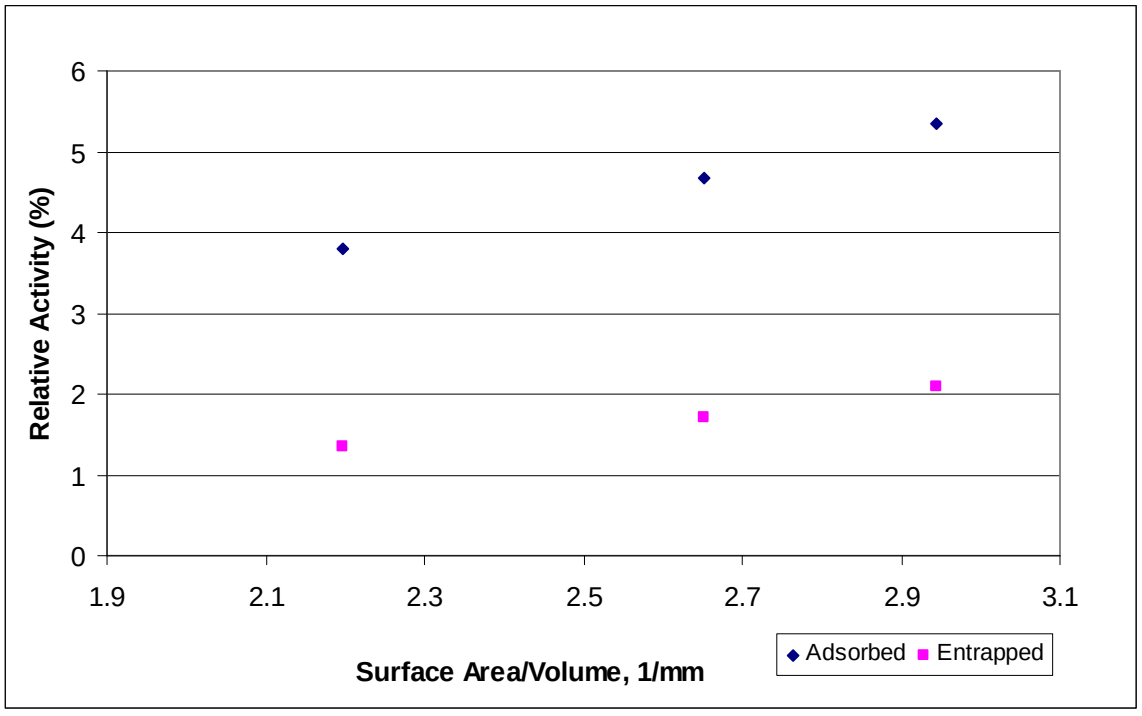

Figure 14: Immobilized enzyme activity/free enzyme activity as a function of surface area to volume ratio

The bead diameters tested has relatively small range (2.04-2.74 mm). Therefore, some of the trends observed for immobilized enzyme activity dependence on bead size appears linear in the bead size range. Testing beads with diameters beyond the smallest and largest beads would allow for further understanding of the effect of particle size on immobilized activity. However, there are limitations to production of alginate bead sizes. Figure 15 shows the calcium alginate bead diameter as a function of the inner diameter of the hypodermic needle used. It is apparent that the smallest bead diameter using this method would be limited by the available hypodermic needles available. Even a 33-gauge needle, the smallest available, has an inner diameter of 0.089 mm and would produce a bead of 1.83 mm calculated based on the extrapolation of the fitted equation to the data.

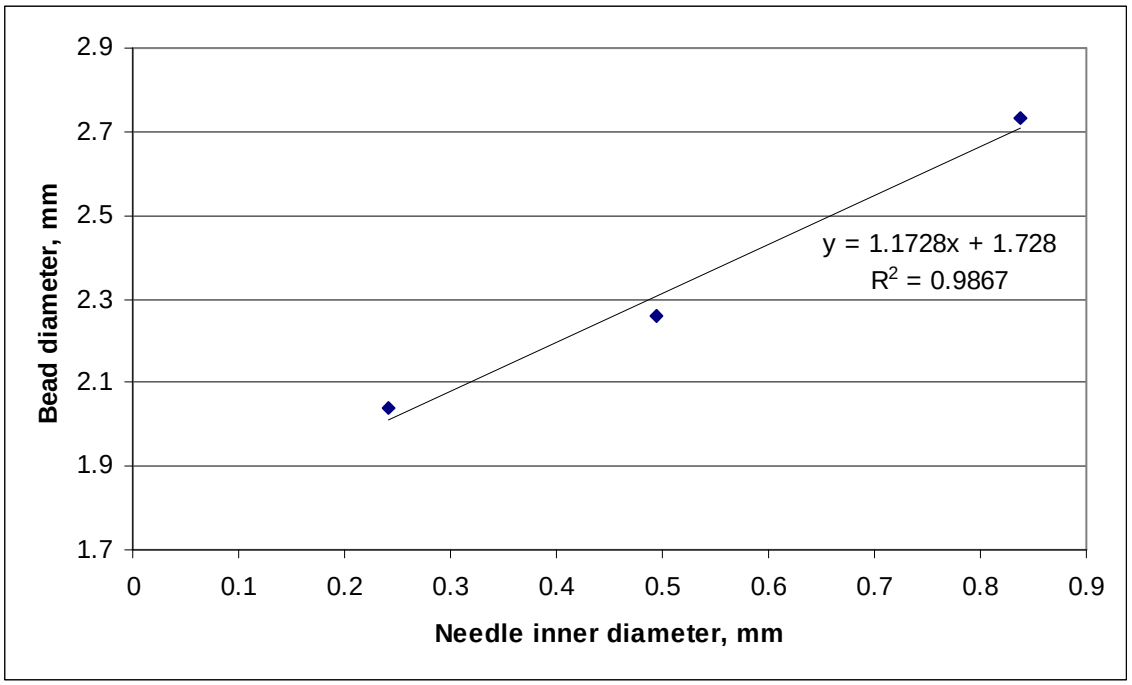

Figure 15: Calcium alginate bead diameter as a function of hypodermic needle inner diameter

Furthermore, as the inner diameter of the hypodermic needle decreases, the pressure drop necessary to maintain the desired flow rate of 1mL/minute would increase according to the Hagen Poiseuille equation:

$$\Delta P = \frac{32\,\mu \cdot L \cdot \nu}{D^2}$$

Where ΔP is pressure drop, μ is viscosity, L is the length of the needle, ν is the velocity, and D is the inner diameter of the needle. A decrease in diameter would cause a much greater increase in the pressure necessary to pass sodium alginate, a viscous liquid, through the needle. Using 2% sodium alginate solution and 5/8 inch 33-gauge needle, ΔP is 113.6 Pa. Table 3 shows the ΔP required for each needle used and the 33-gauge needle.

Table 3: Pressure drop required for various gauge needles using 2% w/v alginate at 25 C

Needle Gauge	18	21	26	33
ΔP (Pa)	1.28	3.67	15.49	113.62

A comparison of the difference in enzyme loading between the two immobilization methods shows that surface adsorption results in greater number of enzymes loaded of alpha-amylase despite the fact that only the surface of the bead is available for binding sites (Table 4). Internal entrapment provides binding sites within the bead's gel matrix as well as the surface thus one would expect higher binding efficiencies with entrapment method.

Table 4: Enzyme loading of both immobilization methods after three trials

Enzyme Loading (%)			
Bead Diameter	2.04	2.26	2.74
Entrapped	46.94 ± .039	38.15± .11	37.97± .044
Adsorbed	64.46 ±.0028	60.74 ± .081	68.76 ± .023

In the entrapment method, alpha-amylase is expected to be immobilized on the surface of the beads as well as be contained inside the beads because the beads are incubated for 1.5 hours in 0.1 M calcium chloride solution to allow the beads to harden. One difference between the two immobilization methods is in relative concentrations of enzyme and calcium during this incubation time. During incubation of enzyme and beads for surface adsorption, the enzyme concentration/calcium concentration ratio is 4800 where as during incubation and hardening of entrapped enzyme the enzyme concentration/calcium concentration is 50. When the beads are formed in calcium chloride, calcium ions displace sodium ions and form a gel matrix. The alpha-amylase that would be immobilized on the bead surface is competing with the calcium ions to move toward the beads and this may deter some surface binding of alpha-amylase from occurring. During the incubation period of the entrapment process, the calcium chloride concentration is 0.1 M, much higher than the 0.005 M calcium chloride presence during incubation of the enzyme with beads for the surface adsorption method. The concentration of alpha-amylase in entrapment incubation environment is about .0123 mg solids/mL while surface adsorption alpha-amylase concentration is .0588 mg solids/mL. Both of these differences in concentration, higher calcium concentration combined with lower alpha-

amylase concentration may have caused fewer enzyme loaded for the internal entrapment method.

5. Conclusions

The hydrolysis of starch using alpha-amylase immobilized by internal entrapment and surface adsorption was the focus of this study. The tests conducted proved that the hypotheses formulated for the study are correct. Surface adsorption produces higher relative activity yield based on free enzyme added than entrapment method. The highest immobilized enzyme activity is obtained with the smallest bead tested for both immobilization methods. The result suggests the dependence of immobilized enzyme activity on surface to volume ratio of the beads. Greater bead surface area in the reaction environment allows for higher enzyme activity and higher relative enzyme activity based on free enzyme activity. Therefore, it is clear that the surface of the beads participated in the starch hydrolysis reaction more readily than the enzyme entrapped within the inner calcium alginate matrix or volume of the beads.

The entrapped enzymes showed lower relative enzyme activity than immobilization of enzyme using surface adsorption. This shows that fewer immobilized alpha-amylases were active with this method because of the substrates' inability to access the enzymes immobilized within the calcium-alginate gel matrix.

All of the reported results are only for only one initial enzyme concentration. The enzyme loading as a function of different enzyme concentrations may display different behaviors and should be explored.

6. Future Recommendations

In this study the effects of bead size and immobilization method were studied. However, the economical use of enzymes requires both high yield of enzyme loading and retention of enzyme activity after reuse. If the enzyme loading or relative activity is low reusability becomes an important factor to increase the yield of reaction per amount of enzyme. Therefore, future studies should test the reusability of the alpha-amylase immobilized using both immobilization methods. This will allow for the most economical immobilization to be implemented by starch processing plants.

All quantitative enzyme analysis for this study utilized the measurement of enzyme activity. Enzyme loading was calculated based on the enzyme activity of spent solutions. If spent solution somehow deactivates enzyme activity the activity tests would give false results. It is possible that enzymes were present but not active in some solutions, therefore protein tests should be used to study the amount of protein present in the spent solution as well as immobilized. This will reduce the error and variability present when testing for enzyme presence based solely on enzyme activity.

A study to test the kinetic parameters of both methods of immobilization would also be of great use to further understand how entrapment and surface absorption affect the maximum velocity (Vmax) of alpha-amylase and it's affinity for the substrate (Km). With knowledge of the kinetic effects of entrapment and surface adsorption, future

research may find ways to increase immobilized enzyme activity similar to that of free alpha-amylases.

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Appendix A: Calibration Curves

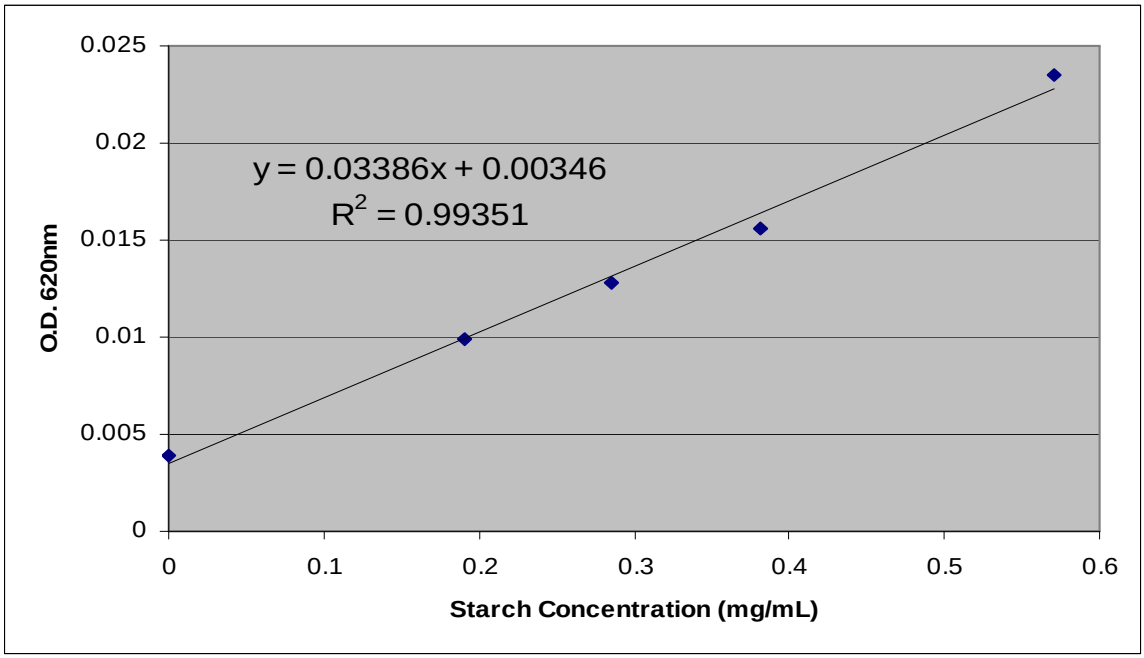

Figure A- 1: Starch calibration curve used to convert absorbance readings to starch concentrations based on colorimetric iodine assay

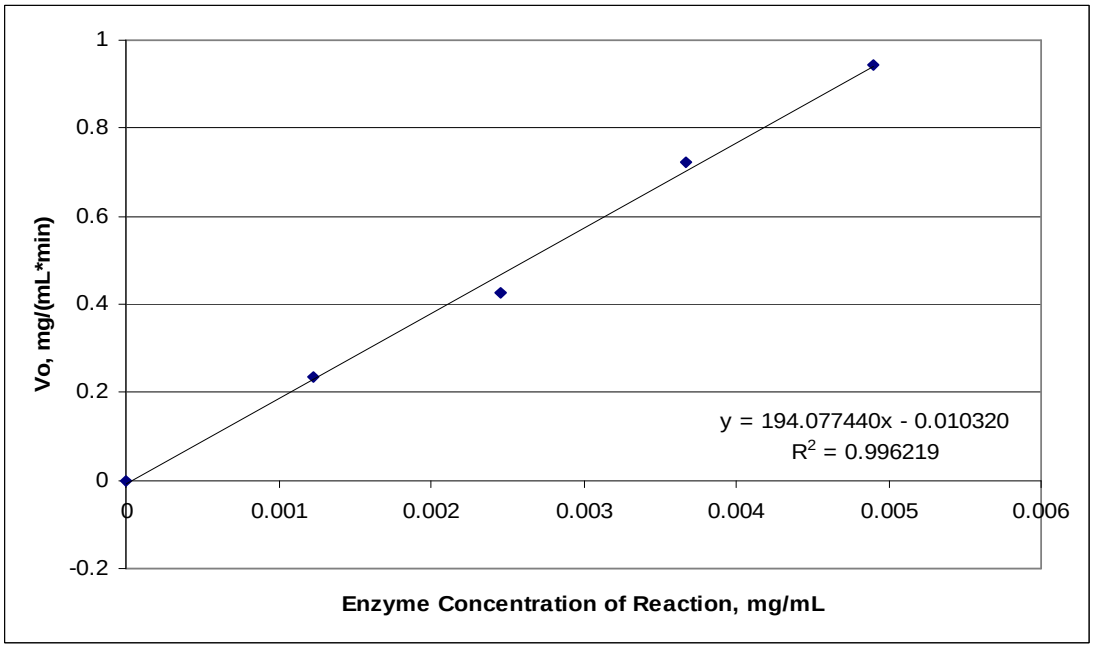

Figure A- 2: Enzyme calibration curve used to convert initial velocity to enzyme concentrations based on colorimetric iodine assay. Starch concentration is 0.57 mg/mL and pH 5.6 for all reactions